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Failure of Phenobarbital to
Induce Rat Hepatic Microsomal
UDP-Glucuronyltransferase toward Phenolphthalein

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FAILURE OF PHENOBARBITAL TO INDUCE RAT HEPATIC
MICROSOMAL UDP-GLUCURONYLTRANSFERASE TOWARD PHENOLPHTHALEIN

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Phenobarbital pretreatment was found not to induce Triton X-100 activated hepatic microsomal neonatal UDP-glucuronyltransferase activity towards phenolphthalein in male, female and pregnant female rats. This was not due to an insufficient concentration of the activating detergent or the choice of detergent. Other microsomal enzymes such as cytochrome P-450 were greatly induced by phenobarbital in the same experimental animals. Late fetal UDP-glucuronyltransferase activity induction by polycyclic hydrocarbons (β -naphthoflavone) was confirmed in this study. Phenobarbital inducibility of neonatal UDP-glucuronyltransferase may not be useful in discriminating between UDP-glucuronyltransferase isoenzymes.

Hepatic microsomal UDP-glucuronyltransferase is a heterogenous group of enzymes responsible for conjugating various endogenous (e.g., bilirubin, estrogen, testosterone) and exogenous (e.g. morphine, chloramphenicol) chemicals to glucuronides and hastening excretion. On the basis of differential induction with phenobarbital and 3-methylcholanthrene there are at least 2 different forms of UDP-glucuronyltransferase (1). However, both UDP-glucuronyltransferase isoenzyme activities have been induced by either phenobarbital or 3-methylcholanthrene (or other aromatic hydrocarbons such as β -naphthoflavone) if the dose is high enough or the pretreatment period long enough (2,3). Thus, Wishart *et al.* (3) proposed that UDP-glucuronyltransferase isoenzymes be called late fetal¹ and neonatal UDP-

¹In this report the terms late fetal and neonatal UDP-glucuronyltransferase refer to different isoenzyme forms, and not to enzyme source (see Table 1). All experimental animals used in these studies were adults.

ABBREVIATIONS UDP, uridine diphosphate

Table 1
Proposed UDP-Glucuronyltransferase Isoenzyme Characteristics^a

	Late fetal isoenzyme	Neonatal isoenzyme
Maximal Activity in Ontogeny	Late fetal	Neonatal
Representative Substrates	p-nitrophenol 1-naphthol 2-aminophenol 2-aminobenzoate 4-methylumbelliferone 5-hydroxytryptamine	phenolphthalein bilirubin testosterone estrogen chloramphenicol morphine
Xenobiotic Induction	3-methylcholanthrene 8-naphthoflavone 2,3,7,8-tetrachlorodibenzo-p-dioxin	phenobarbital
Glucocorticoid Induction	yes	no
Predominant Location in Endoplasmic Reticulum	rough	smooth

^aadapted from Wishart (3,4)

glucuronyltransferase on the basis of ontogenetic differences. These two different forms of UDP-glucuronyltransferase can also be distinguished on the basis of i) glucocorticoid inducibility, ii) substrate specificity and iii) subcellular distribution (3,4) (Table 1).

Other nomenclatures for UDP-glucuronyltransferase heterogeneity have been proposed. Lucier and McDaniel (5) classify UDP-glucuronyltransferase activity into 'steriodal' and 'nonsteriodal' groups which correspond to Wishart's neonatal and late fetal classes, respectively. Classification of UDP-glucuronyltransferase isoenzymes on the basis of biochemical purification and characterization studies have been reviewed recently by Burchell (6).

During xenobiotic induction studies in gestational day 14 pregnant rats, it was observed that phenobarbital pretreatment did not increase UDP-glucuronyltransferase activity for the neonatal substrate phenolphthalein (Kitchin and Ebron, unpublished observations). As pregnant rats are normally more refractory to enzyme induction than nonpregnant females (7,8), the present study was undertaken to determine if the lack of UDP-

glucuronyltransferase induction was due to the pregnancy state of the phenobarbital treated rat (e.g., endogenous sex steroid repression). Thus, the in vitro rate of glucuronidation of the late fetal substrate p-nitrophenol and the neonatal substrate phenolphthalein was determined in normal and phenobarbital treated male, female and pregnant female rats. The results are interpreted with respect to the usefulness of differential induction by phenobarbital and 3-methylcholanthrene to classify the isoenzymes of UDP-glucuronyltransferase.

METHODS

Adult Sprague Dawley rats (CD strain, females 220-280g, males 375-425g) were obtained from Charles River Laboratories and provided with food and water *ad libitum*. The day sperm were detected in morning vaginal smears of bred females is called day 1 of gestation. Sodium phenobarbital (Mallinckrodt Chem. Co.) was dissolved in 0.9% saline and administered i.p. at a dose of 100 mg/kg/day for 5 days prior to sacrifice. β -Naphthoflavone (Aldrich Chem. Co.) was dissolved in corn oil and given intraperitoneally at a dose of 80 mg/kg/day for 5 days. Pregnant rats were treated from days 9-13 of pregnancy and sacrificed on day 14. Control rats were injected i.p. with saline or corn oil. Rats were sacrificed by decapitation, 5g of liver placed in 20 ml of ice cold 0.02 M Tris-HCl buffer, pH 7.5, containing 1.15 M KCl and washed hepatic microsomes prepared as described by Kitchin and Woods (9). The in vitro rates of the glucuronidation of p-nitrophenol (0.8 mM substrate) and phenolphthalein (0.2 mM substrate) were determined by substrate disappearance using 0.2% Triton X-100 activated microsomes (10). The cofactor UDP-glucuronic acid was used at a concentration of 1.4 mM.

Cytochrome P-450 content was assayed by the method of Omura and Sato (11) using an Aminco DW-2 spectrophotometer. Aminopyrine N-demethylase (12,13) and microsomal protein content (14) were performed by published procedures. Statistical comparisons between saline and phenobarbital pretreated groups were done with the Student's T test (15).

RESULTS

In day 14 pregnant female rats, phenobarbital pretreatment increased hepatic microsomal UDP-glucuronyltransferase activity for p-nitrophenol (late fetal isoenzyme) by 62% but did not increase in vitro phenolphthalein glucuronidation (neonatal isoenzyme). In nonpregnant females phenobarbital pretreatment increased the rate of p-nitrophenol glucuronidation by 78% (late fetal) while phenolphthalein glucuronidation (neonatal) was not induced (Table 2). In male rats phenobarbital pretreatment increased

Table 2
UDP-Glucuronyltransferase Activity

Animals/Pre-treatment	Late fetal (p-nitrophenol)		Neonatal (phenolphthalein)	
	Saline	Phenobarbital	Saline	Phenobarbital
Pregnant female	19.3 $\pm$ 1.6	31.2 $\pm$ 5.1 ^a	10.2 $\pm$ 1.1	10.7 $\pm$ 2.0
Female	40.8 $\pm$ 4.2	71.0 $\pm$ 3.5 ^c	16.8 $\pm$ 1.2	17.4 $\pm$ 1.5
Male	33.1 $\pm$ 3.9	68.4 $\pm$ 7.2 ^b	5.8 $\pm$ 0.3	6.7 $\pm$ 0.8

Pregnant female, female and male rats were given saline or phenobarbital (100 mg/kg/day for 5 days) and sacrificed on three different experimental days. Pregnant female rats were treated with phenobarbital during days 9-13 of gestation and sacrificed on gestational day 14. Hepatic microsomal UDP-glucuronyltransferase activity (nmole/mg protein/min) towards representative late fetal (p-nitrophenol) and neonatal (phenolphthalein) substrates were determined. Data is presented as the mean $\pm$ SEM for 5-7 determinations. Statistical significance of phenobarbital versus saline comparisons are given:

^ap<.05
^bp<.01
^cp<.001

late fetal UDP-glucuronyltransferase by 106% but did not increase neonatal UDP-glucuronyltransferase activity.

Possible explanations for the lack of neonatal UDP-glucuronyltransferase by phenobarbital are differences in enzyme latency or the requirement for detergent activation. Thus higher concentrations of Triton X-100 (0.4, 0.6%) were used to activate microsomes from normal and phenobarbital pretreated male rats. In these studies no phenobarbital induction of neonatal UDP-glucuronyltransferase was observed. In further studies the

Table 3
Phenobarbital Induction of Microsomal Cytochrome P-450 and Aminopyrine N-demethylase in Male Rats

	Cytochrome P-450 (nmole/mg protein)	Aminopyrine N-demethylase (nmole/mg protein/min)
Saline	0.51 $\pm$ 0.03	2.93 $\pm$ 0.25
Phenobarbital	1.64 $\pm$ 0.09 ^a	5.97 $\pm$ 0.19 ^a

Male rats were given saline or 100 mg/kg/day phenobarbital for 5 days and sacrificed. Data is the mean $\pm$ SEM for 4-6 independent observations.

^ap<.001

detergent digitonin was used to activate male rat hepatic microsomes obtained from saline or phenobarbital pretreated animals but again no pattern of phenobarbital induced neonatal UDP-glucuronyltransferase activity toward phenolphthalein was observed.

To ensure that the administered phenobarbital was inducing other hepatic microsomal enzymes, the cytochrome P-450 content and aminopyrine N-demethylase activities were assayed in male rats. Substantial oxidative microsomal enzyme induction occurred after pretreatment with 100 mg/kg/day of phenobarbital (Table 3) in the same male rats in which no neonatal UDP-glucuronyltransferase induction was observed (Table 2). Cytochrome P-450 content was increased three fold and aminopyrine N-demethylase activity was doubled by prior administration of phenobarbital.

Finally β -naphthoflavone (an aromatic hydrocarbon inducer of the 3-methylcholanthrene class) was administered to pregnant rats to verify the induction of late fetal UDP-glucuronyltransferase. Late fetal glucuronyltransferase activity towards p-nitrophenol was induced 350% ($P < .001$) while neonatal glucuronyltransferase activity towards phenolphthalein was increased 46% ($P < .05$), the only statistically significant increase in neonatal UDP-glucuronyltransferase activity observed in these studies.

DISCUSSION

The results of these studies confirm the work of Wishart (3) concerning the xenobiotic inducibility of late fetal UDP-glucuronyltransferase.

β -Naphthoflavone, a 3-methylcholanthrene-like inducer, strongly induces p-nitrophenol glucuronidation (late fetal isoenzyme). The effect of phenobarbital on late fetal UDP-glucuronyltransferase were not so dramatic, though still statistically significant effects (Table 2).

However, in our experiments with neonatal glucuronyltransferase no phenobarbital induction could be demonstrated. This disagrees with the work of Wishart (3) in which digitonin was used to activate microsomes

obtained from Wistar rats. In the study of Vaisman et al., (16), phenobarbital increased UDP-glucuronyltransferase activity towards bilirubin (a neonatal substrate) by 15 to 70% in digitonin activated microsomes from nonpregnant and pregnant Sprague-Dawley rats. In pregnant rats, the degree of induction decreased with increasing gestational age. In our studies with Sprague-Dawley rats neither Triton X-100 (0.2, 0.4 and 0.6%) nor digitonin activation revealed any phenobarbital induction of neonatal UDP-glucuronyltransferase. This evidence argues against the possibility that neonatal induced UDP-glucuronyltransferase activity is more latent than uninduced enzyme and requires a greater detergent concentration to fully activate the enzyme activity. The most likely cause of these discrepancies is the different strain of rats used (Wistar versus Sprague-Dawley, CD) or the different neonatal substrate chosen (bilirubin versus phenolphthalein) in these three studies. In our studies in pregnant rats β -naphthoflavone slightly induced UDP-glucuronyltransferase activity for phenolphthalein. Pretreatment of female rats with 2,3,7,8-tetrachlorodibenzo-p-dioxin did not increase UDP-glucuronyltransferase activity for any of the 3 neonatal substrates (phenolphthalein, testosterone or estrogen) examined. As 2,3,7,8-tetrachlorodibenzo-p-dioxin (3 μ g/kg dose) is a much more potent microsomal enzyme inducer than β -naphthoflavone (80,000 μ g/kg dose), membrane mediated effects on UDP-glucuronyltransferase are much less likely with the more potent inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin (17).

The level of induction of neonatal UDP-glucuronyltransferase observed by Wishart (3) was only 60-70% for four out of the six substrates examined (phenolphthalein, chloramphenicol, morphine, bilirubin, testosterone and estradiol). In vitro glucuronidation of chloramphenicol and morphine were found to be most inducible by phenobarbital pretreatment (120% and 180% increases respectively) (3). Thus, either chloramphenicol or morphine might be better marker substrates for neonatal UDP-glucuronyltransferase than phenolphthalein. Neonatal and late fetal UDP-glucuronyltransferase isoenzymes can be separately identified by the criterion of

dose dependent xenobiotic induction with aromatic hydrocarbons such as *o*-naphthoflavone. But the differential xenobiotic induction by phenobarbital may not clearly distinguish between neonatal and late fetal UDP-glucuronyltransferase isoenzyme activities.

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DISCLAIMER

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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